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Preparation of linear maltodextrins using a hyperthermophilic amylopullulanase with cyclodextrin- and starch-hydrolysing activities

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ABSTRACT

A novel method for the preparation of linear maltodextrins from cyclodextrins and starch was proposed. To accomplish this process, an amylopullulanase from hyperthermophilic archaeon *Caldivirga maquilingensis* (CMApu) was characterized and used. CMApu with an estimated molecular mass of 62.7 kDa by SDS-PAGE had a maximal pullulan-hydrolysing activity at 100 °C and pH 5.0. It could also hydrolyse amylopectin (AP), starch, β-CD and amylose (AM), in a decreasing order of relative activities from 88.96% to 57.17%. TLC and HPAEC analysis revealed that CMApu catalyzed the debranching and degrading reactions to produce linear malto-oligosaccharides (≤G8–G1) from G8-β-CD and/or normal CDs, amylodextrins (DP6–96) from AM, and amylodextrins (DP1–76) from AP and potato starch. Our results showed that CMApu had a great potential for the industrial preparation of linear maltodextrins from normal starch instead of waxy starch, malto-oligosaccharides or sucrose. And the high optimal temperature of CMApu facilitated the simultaneous gelatinization and hydrolysis of cereal starch.

1. Introduction

Maltodextrins with the properties of moderate sweet, rapidly absorption and hygroscopicity are widely used in numerous foods (Chronakis, 1998; Fisher-Wellman & Bloomer, 2010; Kendig, Lin, Beilharz, Rooney, & Boakes, 2014). Linear maltodextrins, composed of a mixture of polymers of D-glucose units with α-1,4-glucosidic linkages, were classified by degree of polymerization (DP) into linear malto-oligosaccharides (DP ≤ 8) and amylodextrins (1 ≤ DP < 17 or more long). Compared with branched malto-oligosaccharides with α-1,6-glucosidic linkages besides α-1,4-glucosidic linkages, linear malto-oligosaccharides are more easily hydrolysed by human salivary or pancreatic α-amylase and mucosal α-glucosidase resulting in higher glycaemic index for nutrition and energy. Amylodextrins could reduce the amylose recrystallization of cereal foods (Xu et al., 2012) and improve the stability of ω3/ω6 polyunsaturated fatty acids (Xu et al., 2013). They were non-swelling, insoluble, and usually included in the ingredient of tablets for a controllable release rate of drug (Steendam, Eissens, Frijlink, & Lerk, 2000; TeWierik, Eissens, Besemer, & Lerk, 1993).

Linear maltodextrins were often prepared by enzymatic hydrolysis of amylose, amylopectin, malto-oligosaccharides or transglycosylation of sucrose. These amylolytic enzymes, with different activities and action patterns, were classified into different families of glycoside hydrolases (GH) (Janeček, Svensson, & MacGregor, 2014). Human saliva, porcine pancreas, or *Bacillus subtilis* α-amylase of GH family 13 could attack the α-1,4-glucosidic linkages of amylose at 37–60 °C to produce amylodextrins directly (Jane & Robyt, 1984). The desired DP could be obtained by adding different amount of α-amylase in the reactions (Xu et al., 2012). *Bacillus acidopullulyticus* type I pullulanase of GH family 13 could specifically attack the α-1,6-glucosidic linkages of amylopectin. When it was incubated with waxy maize starch at 55 °C, the amylodextrins with a DP of 35 were formed by the debranching reaction of amylopectin (TeWierik, Eissens, Besemer, & Lerk, 1993). *Escherischia coli* amylomaltase (Pajatsch, Böck, & Boos, 1998) and *Saccharophagus degradans* 4-α-glucanotransferase (Hwang, Choi, Kim, & Cha, 2013) of GH family 77 at 35 °C could catalyse the disproportion reaction between maltose, maltotriose or maltodextrins

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to synthesize the longer linear maltodextrins. *Neisseria polysaccharea* amylosucrase of GH family 13 at 35 °C could catalyse the formation of linear maltodextrins with a DP of 35–48 by successive transfers of the glucosyl moiety of sucrose onto the released glucose in the presence of sucrose alone on the initial concentration of 600–100 mM (Potocki-Veronese et al., 2005). More recently, rabbit muscle phosphorylase-a was used at 37 °C to prepare linear maltodextrins with a DP of 6–89 by successive transfers of the glucosyl moiety of a-D-glucose 1-phosphate onto maltopentaose, maltohexaose, or maltoheptaose (Kazłowski & Ko, 2014). However, it is not suitable for these amylolytic enzymes to produce linear maltodextrins from normal cereal starch.

Amylopullulanase, a type II pullulanase, with the cleavage activity of both α-1,4- and α-1,6-glucosidic linkages in starch and other related oligosaccharides, is attractive to prepare the linear maltodextrins using the cheap starch as the only substrate by breaking the chains of amylose and debranching the side chains of amylopectin. Thermostable amylopullulanases are especially favorable in the hydrolysis of starch, as high temperatures often facilitate the simultaneous gelatinization of starch, accelerate the reaction rate, and decrease the possibility of microbial contamination.

Hyperthermophilic archaea with optimal growth temperatures between 80 and 110 °C have been considered as a source of genes encoding thermostable enzymes (Egorova & Antranikian, 2005; Vieille & Zeikus, 2001). The archaeal amylopullulanases of GH family 57 from *Thermococcus hydrothermalis* (Erra-Pujada, Debeire, Duchiron, & O'Donohue, 1999; Gantelet & Duchiron, 1998), *T. litoralis* (Imamura et al., 2004), *T. siculi* (Jiao et al., 2011), *Pyrococcus furiosus* (Dong, Vieille, & Zeikus, 1997; Rüdiger, Jorgensen, & Antranikian, 1995) and *Staphylothermus marinus* (Li, Li, & Park, 2013) have been cloned and expressed in mesophilic *E. coli* successfully. All these recombinant enzymes have the maximal activities at their optimal temperatures between 85 and 105 °C, indicating the extremely thermostable properties.

Caldivirga maquilingensis is an anaerobic, sulfur-dependent, rod-shaped, and hyperthermoacidophilic Crenarchaeota isolated from an acidic hot spring in the Philippines (Itoh, Suzuki, Sanchez, & Nakase, 1999). This microorganism has a high amount of tetraether core lipids and trace amounts of diether core lipids to help it thrive within its very hot (60–92 °C) and very acidic (pH 2.3–6.4) environment. Genomic sequencing of *C. maquilingensis* has revealed that its some genes might be related to the hydrolysis of starch and transportation (Copeland et al., 2007).

In this study, a gene encoding a protein of GH family 57 from *C. maquilingensis* was cloned and expressed in *E. coli*. Characterization of the biochemical properties of the purified recombinant protein revealed that it was an extremely thermostable amylopullulanse with CD/AM-degrading and long branched cyclodextrin (LBCD)/AP-debranching activities. The results provided a novel method for the production of linear maltodextrins from LBCD or normal cereal starch.

2. Materials and methods

2.1. Chemicals

Pullulan, potato starch (normal starch, amylose, and amylopectin), malto-oligosaccharides (from maltose to maltoheptaose; i.e. G2–G7), cyclodextrins (α-CD, β-CD, and γ-CD), and short branched cyclodextrins (6-*O*-glucosyl-β-CD and 6-*O*-maltosyl-β-CD; i.e. G1-β-CD and G2-β-CD) were purchased from Sigma (St. Louis, MO, USA). Long branched cyclodextrin (6-*O*-maltooctaosyl-β-CD; i.e., G8-β-CD) was kindly provided by Professor Kwan-Hwa Park (Department of Foodservice Management and Nutrition, Sangmyung University, Republic of Korea). All restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase, and PrimeSTAR® HS DNA Polymerase were supplied by Takara Biotechnology (Dalian, Liaoning, China). All other chemicals used were of reagent grade and were purchased from Sinopharm Chemical (Shanghai, China). Double distilled water was prepared with a distilled water system (BSZ-2; Botonyc, Shanghai, China). Deionized water was prepared with an ultrapure water system (Molelement 1820a; Molecular, Shanghai, China).

2.2. Cloning and expression of the gene encoding CMApu

The gene encoding CMApu was amplified from *C. maquilingensis* IC-167 genomic DNA (kindly provided by Professor Christopher Howard House, Department of Geosciences, Pennsylvania State University, USA) by PCR using PrimeSTAR® HS DNA Polymerase at an annealing temperature of 55 °C (G-Storm Thermal Cycler; GRI, Braintree, Essex, UK). CMApu gene-specific primers flanking the 5′ and 3′ ends of Cmaq_1371 were designed based on the complete genome sequence of *C. maquilingensis* IC-167 (Copeland et al., 2007). The forward (Cmaq_1371-*Nde*I, 5′-CATTCATATGGTCTACGTGAGGGCTTTACTTGATG-3′) and reverse (Cmaq_1371-*Xho*I, 5′-AGCCTCGAGCGCTGAATCCTCATTAGGT-3′) primers (Takara Biotechnology) contained *Nde*I and *Xho*I restriction sites (underlined), respectively. The amplified fragment (1.82 kb) was digested with *Nde*I and *Xho*I, and then ligated into the expression vector pTKNd6xH (kindly provided by Professor Kwan-Hwa Park) with a BLMA promoter (Kim et al., 2003). The recombinant plasmid was designated pCMApu6xH. The sequence of inserted Cmaq_1371 was confirmed using dideoxy chain termination sequencing with an Applied Biosystems® 3730xl DNA Analyzer (Life Technologies, Foster City, CA, USA).

2.3. Production and purification of recombinant CMApu

E. coli MC1061 cells transformed with pCMApu6xH were cultured for 20 h at 250 rpm 37 °C in 6 flasks containing 250 mL of Luria-Bertani broth [1% (w/v) tryptone (Oxoid, Basingstoke, Hampshire, UK), 0.5% (w/v) yeast extract (Oxoid), and 0.5% (w/v) NaCl] supplemented with kanamycin (80 μg/mL). The cells were then collected by centrifugation at 9000 × *g* and 4 °C for 20 min (Avanti® J-26XP Centrifuge; Beckman Coulter, Brea, CA, USA), resuspended in 150 mL of binding buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, and 5 mM imidazole), and sonicated 5 times for 3 min each for 50 mL each of resuspended *E. coli* in an ice bath using a ultrasonic processor with a 1/2″ (13 mm) probe with replaceable tip at net power output 750 W, frequency 20 kHz, amplitude 35%, pulse on 2 s and pulse off 1 s (VCX 750; Sonics & Materials, Newtown, CT, USA). The cell-free supernatant (163 mL) was then collected by centrifugation at 12,000 × *g* and 4 °C for 20 min, and then heated at 70 °C for 15 min to remove all thermolabile proteins. The crude enzyme from heat treatment (154 mL) was further purified by an ÄKTApurifier UPC 10 system with a Ni Sepharose™ 6 Fast Flow column (2.6 cm × 10 cm) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The active fractions in elution buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, and 500 mM imidazole) were concentrated and dialyzed by ultrafiltration (Amicon stirred cell model 8400; Merck Millipore, Billerica, MA, USA) against 20 mM sodium phosphate buffer (pH 7.4). The protein concentration was determined according to the Bradford method, using bovine serum albumin as a standard (Bradford, 1976). The purity and molecular weight of the protein were analysed by discontinuous SDS-PAGE (Laemmli, 1970).

2.4. Enzyme assay

The activities of CMApu for pullulan, β-CD, starch, amylose and amylopectin were assayed at 100 °C in 50 mM sodium acetate buffer (pH 5.0) with 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Substrate (1% (w/v), 150 μL), 75 μL of 200 mM sodium acetate buffer (pH 5.0) and 45 μL of double distilled water were mixed and preincubated at 100 °C in a digital dry bath (MyBlock™ HL; Benchmark Scientific, Inc., Edison, NJ, USA). The enzyme (30 μL) was added to the preheated solution, and then the reaction mixture was incubated for 10 min. The reaction was stopped by adding 900 μL of 3,5-dinitrosalicylic acid solution, then boiling for 5 min, and finally cooling with iced water. The absorbance of the reaction mixture (200 μL) at 575 nm was measured using a microplate reader (Multiskan Spectrum; Thermo Scientific, Vantaa, Finland). The amount of reducing sugars released from substrates was calculated with maltose standard curve. By DNS method, one unit (U) of enzymatic activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugars per minute.

2.5. Analysis of the influence of the pH and temperature on CMApu activity

The optimal pH was determined by examining the relative activity of CMApu (0.1 U) toward pullulan (0.5%, w/v) at 100 °C in 300 μL of 50 mM sodium acetate buffer (pH 4.0–5.5) and 50 mM sodium phosphate buffer (pH 6.0–7.0). The optimal temperature was determined by examining the relative activity of CMApu (0.1 U) toward pullulan (0.5%, w/v) in 300 μL of 50 mM sodium acetate buffer (pH 5.0) at a temperature range from 80 °C to 105 °C in a digital dry bath. The reactions were carried out for 10 min.

2.6. Hydrolysis of various substrates by CMApu

α-CD, β-CD, γ-CD, G1-β-CD, G2-β-CD, G8-β-CD, G3 and acarbose were used as the substrates of CMApu to examine the hydrolytic patterns. A-50 μL of CMApu (0.8 U) was incubated with 125 μL of 1% (w/v) substrate, 62.5 μL of 200 mM sodium acetate buffer (pH 5.0) and 12.5 μL of double distilled water at 95 °C for various time. The hydrolytic products were analysed by thin-layer chromatography (TLC).

Normal starch, amylose, and amylopectin from potato were used as the substrates of CMApu to prepare the linear maltodextrins. To obtain 1% (w/v) amylose solution, amylose was dissolved with 1 M NaOH, then diluted 10 times with double distilled water, and finally neutralized with 0.1 M HCl (Park et al., 2007). For the hydrolysis of amylose, a-10 μL of CMApu (0.16 U) was incubated with 200 μL of amylose, 250 μL of 200 mM sodium acetate buffer (pH 5.0) and 540 μL of double distilled water at 100 °C for 1 h. For the hydrolysis of amylopectin or starch, a-10 μL of CMApu (0.16 U) was incubated with 2 mg of amylopectin or starch, and 90 μL of 50 mM sodium acetate buffer (pH 5.0) at 100 °C for 1 h. The reaction solutions (0.2%, w/v) were passed through a 0.2-μm disposable syringe filter, and then the hydrolytic products were analysed by a high-performance anion exchange chromatography (HPAEC).

2.7. TLC analysis

Silica gel plates (Whatman K5F; GE Healthcare, Maidstone, Kent, United Kingdom) were activated at 110 °C for 30 min. The reaction solutions (2 μL) were spotted on the plate using a pipette, and then developed at room temperature in a TLC chamber containing a solvent mixture of n-butanol/ethanol/water (5:5:3, v/v/v). The plates were dried at room temperature, and dipped into a solution containing 0.3% (w/v) N-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol. The hydrolytic products were visualized by heating the plates at 110 °C for 10 min.

2.8. HPAEC analysis

A HPAEC system with an electrochemical detector containing Au working and Ag/AgCl reference electrodes (ICS-3000; Dionex, Sunnyvale, CA, USA) was used. The reaction solution (Sample loop, 25 μL) was injected into an anion-exchange column (3 mm × 250 mm, CarboPac™ PA200, Dionex) connected with a guard column (3 mm × 50 mm, CarboPac® PA200, Dionex). The temperature of column oven was kept at 30 °C. The mobile phases consisted of eluents A (100 mM NaOH; Alfa Aesar, Ward Hill, MA, USA) and B (1 M sodium acetate, electrochemical grade from Dionex, in 100 mM NaOH) were pumped at a flow rate of 0.4 mL/min. Eluent B was increased linearly from 0% to 60% in 0–60 min (Li, Li, Tian, & Park, 2014). The pulsed amperometry detection was performed in standard quad waveform for carbohydrates. The compositions of linear maltodextrins were characterized as a percentage of the total peak area (Hanashiro, Abe, & Hizukuri, 1996).

2.9. Statistical analysis

SPSS Statistics V22.0 (IBM, Armonk, New York, USA) was used for the statistical analysis. Results were expressed as the mean plus or minus the standard deviation (SD). Statistically significant differences between enzymatic specific activities for different substrates were compared using a Duncan's multiple range test at the 0.01 level.

3. Results and discussion

3.1. Conserved region and sequence homology of CMApu

Standard protein-protein BLAST (blastp) (Altschul & Lipman, 1990; Altschul et al., 1997), provided by the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi), was used for both identifying the CMApu sequence and for finding similar sequences in the database of non-redundant protein sequences. The BLAST results of CMApu suggested that residues 109–432 constituted a GH57N_APU domain of $(\beta/\alpha)_7$-barrel fold including catalytic nucleophile E270, proton donor D366, substrate binding H114, H116, W423, W430, and CMApu had 34.5% maximum sequence identity with *S. marinus* amylopullulanase (Li et al., 2013).

3.2. Overexpression and purification of CMApu

PCR-amplified Cmaq_1371 was ligated into downstream of the *Bacillus licheniformis* MAase (BLMA) promoter of pTKNd6xH to create pCMApu6xH. Recombinant CMApu, with eight additional amino acids [-Leu-Glu-6xHis] at the carboxyl terminus, was expressed under the control of BLMA promoter in *E. coli* harboring pCMApu6xH. The specific activity of CMApu in the crude extract of sonicated cells was 0.7 U/mg of protein. The total activities increased from 177.0 U to 184.8 U after heat treatment at 70 °C for 15 min. The reason for the enhanced enzymatic activities possibly lied in the formation of proper protein folding for CMApu at the high temperature. Recombinant CMApu purified in the absence of heat treatment may be in an intermediate stage of protein folding. This phenomenon has been observed in many thermostable enzymes (Lee et al., 2007). The heat-treated enzyme was finally purified 10-fold to a specific activity of 7.3 U/mg of protein using Ni-Sepharose affinity chromatography (Table 1).

Table 1
Purification of CMApu from a 1.5-L culture of *E. coli*.

Purification step	Total volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell extract	163.0	177.0	255.9	0.7	100.0	1.0
Heat-treated	154.0	184.8	55.4	3.3	104.4	4.8
Ni-Sepharose	2.1	32.3	4.4	7.3	18.2	10.6

The purified recombinant CMApu showed a major band with an estimated molecular mass of 62.7 kDa by SDS-PAGE (Fig. 1, lane 3).

3.3. Biochemical properties of CMApu

To obtain the optimal conditions for CMApu catalysis, the pullulan-degrading reactions were performed at various pH values and temperatures. The optimal pH was observed at pH 5.0 (Fig. 2a). More than 50% of the maximum activity was retained in the range between pH 4.5 and pH 7.0. The optimal temperature was observed at 100 °C (Fig. 2b), and even at 105 °C, 56.6% of the maximum activity was detected. The other tests showed that the optimal temperature of CMApu would decrease at more acidic conditions. Nevertheless, 84.6% of the maximum activity could still be kept at pH 4.0 and 90 °C. These results revealed that the weak acidic pH and water boiling temperature were the optimal catalytic conditions for CMApu same as the other hyperthermophilic archaeal amylopullulanases (Dong et al., 1997; Erra-Pujada et al., 1999; Imamura et al., 2004; Jiao et al., 2011; Li et al., 2013).

To investigate the substrate specificity of CMApu, its hydrolytic activities toward pullulan, starch, amylopectin, amylose, and β-CD were determined. CMApu had the highest level of specificity toward pullulan, followed by amylopectin and starch at 88.96–86.02%, β-CD and amylose at 61.23–57.17%, relative to that using pullulan (Table 2). Duncan multiple comparisons showed that there was no significant difference at the 0.01 level between specific activities for starch and amylopectin, indicating that normal starch was a promising candidate for enzymatic preparation of linear maltodextrins. CMApu could slightly hydrolyse maltotriose and acarbose (a potent α-amylase inhibitor) into maltose plus glucose and acarviosine-glucose plus glucose, respectively. CMApu could never hydrolyse maltose, panose, and isomaltotriose (data not shown).

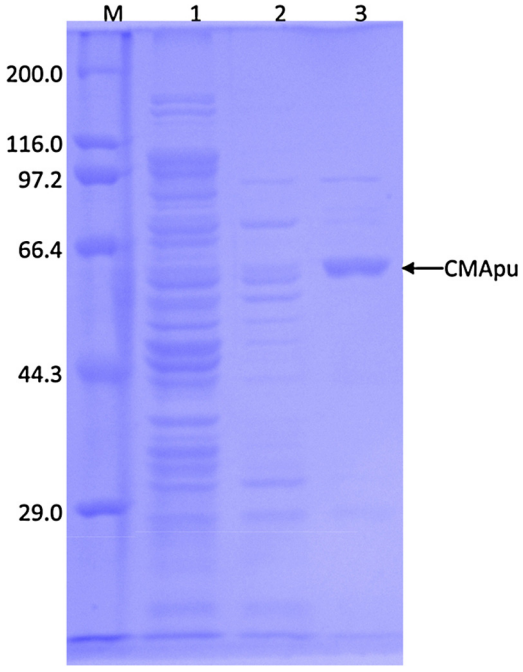

Fig. 1. SDS-PAGE of CMApu at different stages of purification from the *E. coli* lysate. Lane M, protein size standards; lane 1, proteins from the sonicated cells; lane 2, proteins after heat treatment (70 °C, 15 min); and lane 3, purified CMApu after Ni Sepharose column chromatography.

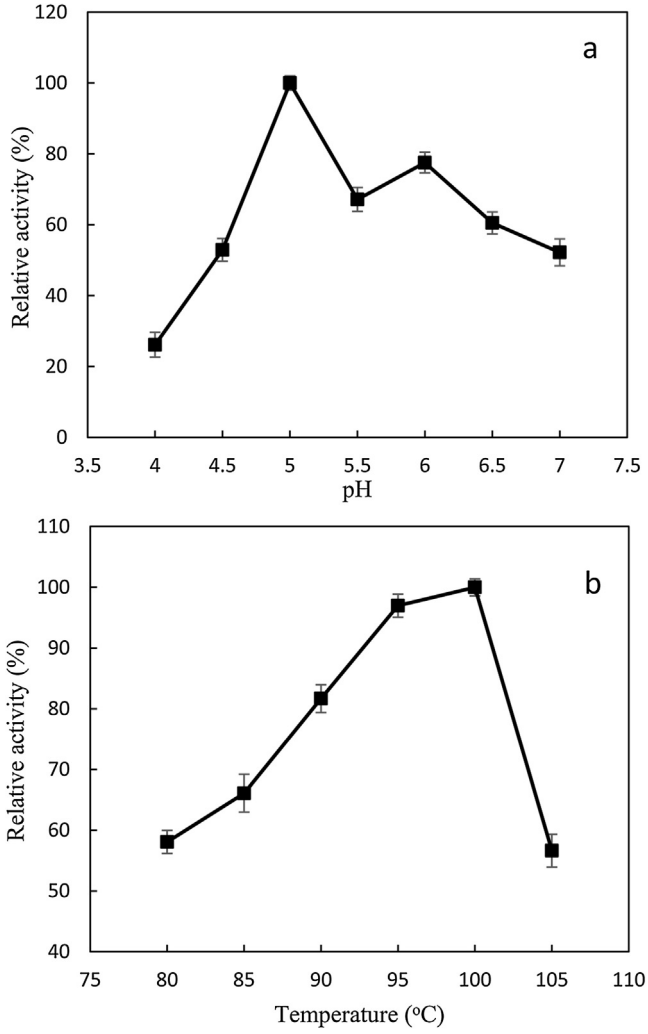

Fig. 2. Effect of pH (a) and temperature (b) on the activity of CMApu. The pH and temperature optima of CMApu were measured using 0.5% (w/v) pullulan as the substrate in 50 mM sodium acetate buffer (pH 4.0–5.5) or 50 mM sodium phosphate buffer (pH 6.0–7.0), at 80–105 °C.

Table 2
Substrate specificity of CMApu. CMApu activity was determined at 100 °C in 50 mM sodium acetate buffer (pH 5.0) using different substrates with DNS method.

Substrate	Specific activity[a] (U/mg)	Relative activity (%)
Pullulan	7.90 ± 0.34a	100.00
Starch	6.80 ± 0.11b	86.02
Amylopectin	7.03 ± 0.29b	88.96
Amylose	4.52 ± 0.24c	57.17
β-CD	4.84 ± 0.18c	61.23

[a] Values were expressed as mean ± SD ($n = 3$). Values sharing the same lowercase letters were not significantly different ($p < 0.01$).

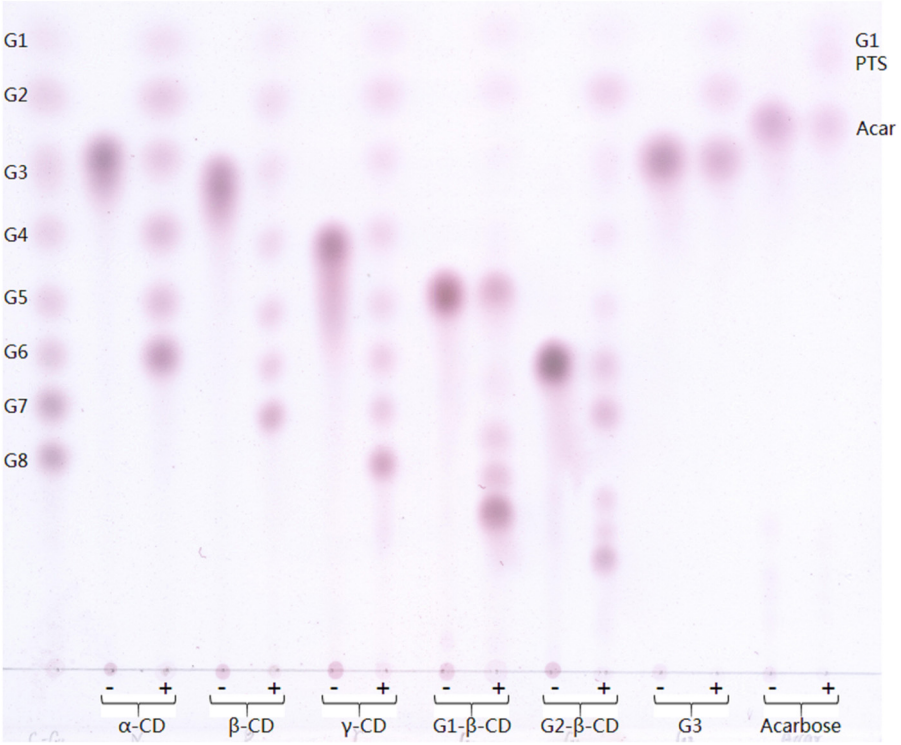

Fig. 3. TLC of hydrolytic products from 0.5% (w/v) various substrates incubated at 95 °C and pH 5.0 for 30 min with (+) and without (−) CMApu (0.64 U/mg substrate). Standard maltooligosaccharides were indicated by G1–G8. Acarbose and acarviosine-glucose were indicated by Acar and PTS, respectively.

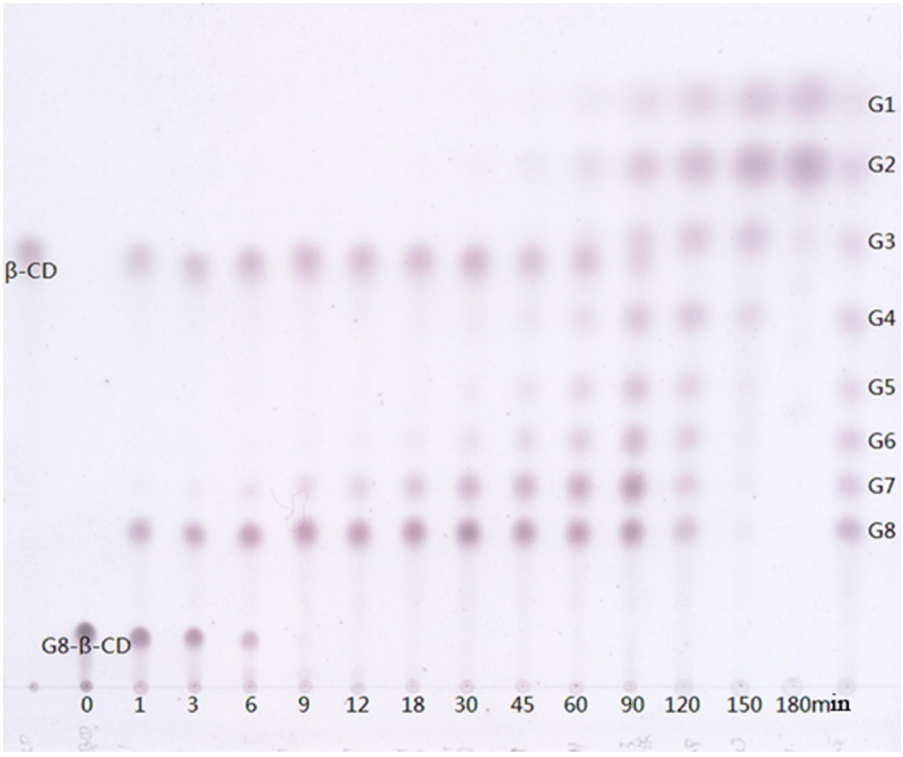

Fig. 4. TLC of hydrolytic products from 0.5% (w/v) G8-β-CD incubated with CMApu (0.16 U/mg substrate) at 95 °C and pH 5.0 for various time. Standard maltooligosaccharides were indicated by G1–G8. Standard β-CD and G8-β-CD were marked under the corresponding spots.

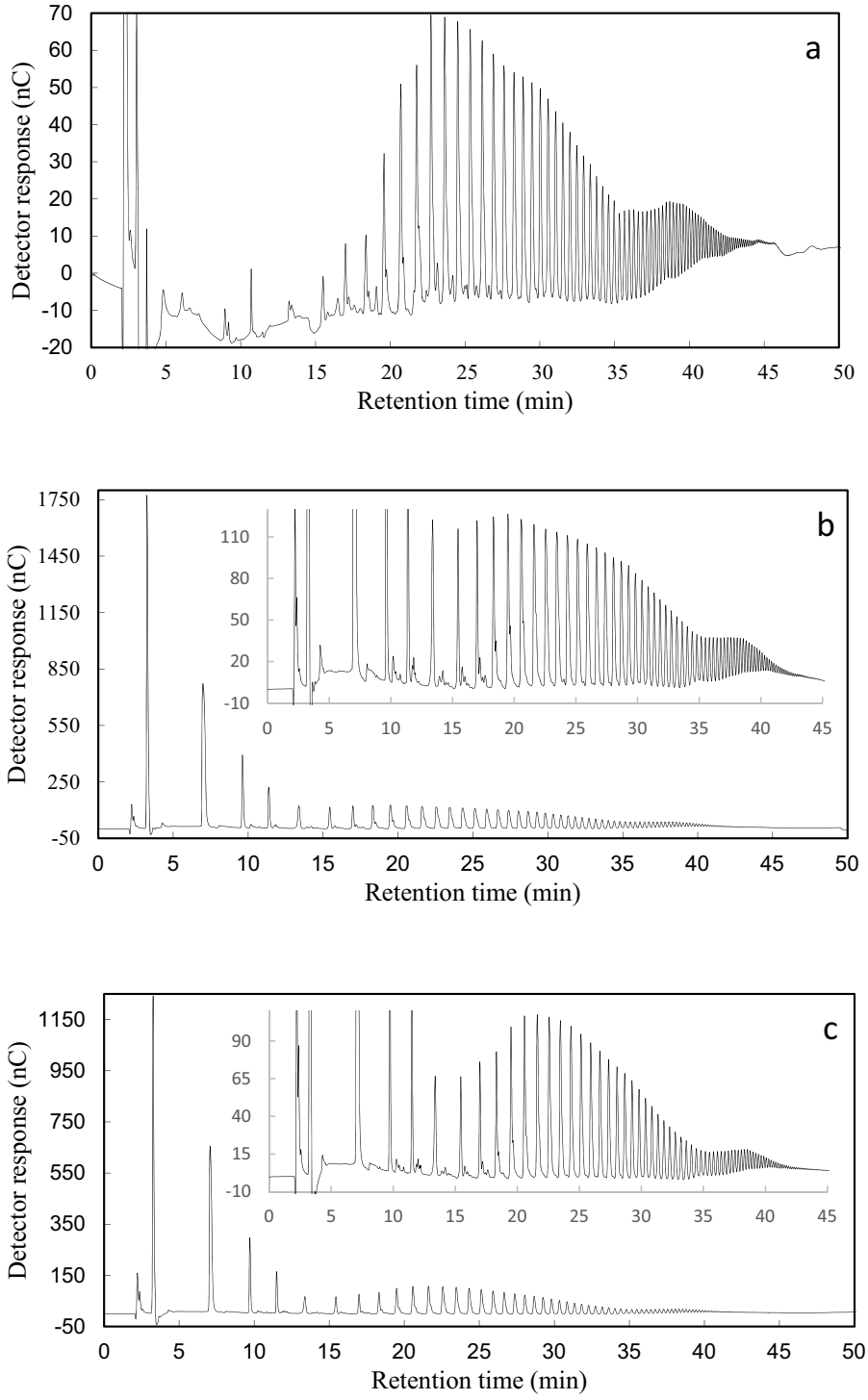

Fig. 5. HPAEC of hydrolytic products from 0.2% (w/v) amylose (a), 2% (w/v) amylopectin (b), and 2% (w/v) starch (c) incubated with CMApu (0.08 U/mg substrate) at 100 °C and pH 5.0 for 1 h. The enlarged partial images were also included in (b) and (c).

Considering pullulan and starch as the optimal substrates, this enzyme from GH family 57 was named as amylopullulanase. It is also the second enzyme after *S. marinus* amylopullulanase (Li et al., 2013) with the cyclodextrin-degrading activity in the GH family 57 (Blesák & Janeček, 2013).

3.4. Preparation of linear malto-oligosaccharides by CMApu

Several cyclodextrins including normal cyclodextrins and branched cyclodextrins were respectively incubated with CMApu at 0.64 U/mg substrate for 30 min. The profile of hydrolytic products was identified by TLC.

TLC chromatogram (Fig. 3) showed that CMApu could hydrolyse α-CD, β-CD, and γ-CD into the corresponding linear malto-oligosaccharides (from maltohexaose to glucose, maltoheptaose to glucose, and maltooctaose to glucose, respectively) at 30 min same as *P. furiosus* thermostable amylase (Yang et al., 2006), *Thermofilum pendens* maltogenic amylase (Li, Li, Yin, & Park, 2010) and *S. marinus* amylopullulanase (Li et al., 2013).

Table 3
Chain length distributions (%) of linear maltodextrins prepared from potato starch using CMApu.[a]

Potato starch	DP < 6	DP6–12	DP13–24	DP25–36	DP ≥ 37	DPw
Amylose	4.37 ± 0.57	17.54 ± 0.42	41.52 ± 0.28	18.41 ± 0.22	18.17 ± 0.91	23.66 ± 0.47
Amylopectin	47.44 ± 0.10	16.56 ± 0.18	21.79 ± 0.46	8.36 ± 0.22	5.86 ± 0.32	11.47 ± 0.14
Normal starch	46.84 ± 0.06	16.87 ± 0.18	24.18 ± 0.40	7.21 ± 0.10	4.89 ± 0.52	11.14 ± 0.22

[a] Values were expressed as mean ± SD (n = 3).

However, CMApu firstly catalysed the ring-opening reactions of G1-β-CD and G2-β-CD, the short branched cyclodextrins, to produce 6^n-*O*-α-glucosyl-maltoheptaose and 6^n-*O*-α-maltosyl-maltoheptaose (kinetic analysis not shown), appeared as the lowest spots in the corresponding reactions (Fig. 3). This revealed that CMApu had an *O*-glucosidic bond cleavage preference for ring-opening over debranching toward short branched cyclodextrin. Then, these two-branched malto-oligosaccharides were partial converted into a mixture of branched and linear malto-oligosaccharides in the reaction conditions same as those of normal cyclodextrins.

Unlike the short-branched cyclodextrins, the TLC chromatogram (Fig. 4) showed that all G8-β-CDs were quickly debranched to form β-CDs and maltooctaoses during the initial stage of the reaction. This revealed that CMApu had an *O*-glucosidic bond cleavage preference for α-1,6-glucosidic linkage over α-1,4-glucosidic linkage toward LBCD. Then, maltoheptaose was produced from the ring-opening reaction of β-CD. At this stage, maltooctaose was not hydrolysed temporarily since no other spot was found on the TLC plate. This indicated that CMApu had a substrate preference for β-CD over maltooctaose. At 120 min, all β-CDs disappeared, and linear malto-oligosaccharides from maltooctaose to glucose were prepared. If the incubation continued to be performed, the linear malto-oligosaccharides were finally hydrolysed into maltose and glucose by the residual activity of CMApu.

Sulfolobus solfataricus debranching enzyme (TreX) (Nguyen et al., 2014; Park et al., 2008), *B. subtilis* pullulanase (AmyX) (Shim et al., 2009) and *S. marinus* amylopullulanase (Li et al., 2013) also possessed the similar debranching modes toward short and long branched cyclodextrins. But TreX and AmyX lacked the ring opening activity of cyclodextrins.

3.5. Preparation of amylodextrins by CMApu

Amylose, amylopectin, and normal starch from potato were respectively incubated at 100 °C with CMApu at 0.08 U/mg substrate for 1 h. The profiles of hydrolytic products were identified by HPAEC. The proportions of chains with different degree of polymerization (DP < 6, DP6–12, DP13–24, DP25–36 and DP ≥ 37) were calculated by a sum of relative peak areas.

HPAEC chromatogram (Fig. 5a) revealed that amylodextrins with a main DP range from 6 to 96 were produced from amylose, suggesting that CMApu usually broke the α-1,4-glucosidic linkages of amylose at intervals of at least six glucosyls. The amylodextrins from CMApu-treated amylose had a higher proportion (41.52%) of DP 13–24 chains (Table 3).

HPAEC chromatogram (Fig. 5b and c) revealed that amylodextrins with a DP range from 1 to 76 were produced from amylopectin and starch. The related reactions might be involved in the cleavages of both α-1,6- and α-1,4-glucosidic linkages since CMApu had the cleavage activity toward these two bonds. Unlike the amylodextrins from CMApu-treated amylose, the amylodextrins from CMApu-treated amylopectin and starch had the similar profile with a higher proportion (47%) of DP < 6 chains (Table 3). The weight-average degree of polymerization (DPw) of the amylodextrins from amylose was two times more than those of the amylodextrins from amylopectin and starch, indicating that amylose was more suitable for the preparation of long amylodextrins.

In the HPAEC chromatogram, the total peak areas of amylodextrins from amylose, starch, and amylopectin in increasing order were correlated to the activities of CMApu toward these substrates. Accordingly, it is necessary to add more enzymes into the reaction for a high yield of amylodextrin especially when amylose is used as the substrate.

The aforementioned results revealed that the amylodextrins could be directly prepared from normal cereal starch at a low cost by breaking the chains of amylose and debranching the side chains of amylopectin, which benefited from the novel catalytic properties of CMApu. And, the feature of CMApu with high optimal temperature of 95–100 °C facilitated the simultaneous gelatinization and hydrolysis of cereal starch. In the boiling or nearly boiling water, hot water enters the semi-crystalline regions of raw starch, breaks down the intermolecular hydrogen bonds, and binds to the hydroxyl hydrogen and oxygen so that the chains of starch lose their crystallinity into the amorphous, separated and hydrated form. CMApu driven by hot water entered the inside of starch granules to attack the chains.

However, the optimal temperatures of some amylolytic enzymes including *Aspergillus niger* amyloglucosidase and *B. acidopullulyticus* type I pullulanase are lower than the gelatinization temperatures of normal cereal starch. During the preparation of linear maltodextrins, the slurries of retrograded or waxy starch are often heated to 100 °C for further gelatinization. Then, an extra cooling step is still required to obtain 40–60 °C for the enzymatic reactions (Andersson, Rydberg, Larsson, Andersson, & Åman, 2002; Wong, Muhammad, Dzulkifly, Saari, & Ghazali, 2007).

4. Conclusions

Based on the investigations of catalytic properties of CMApu, a novel method for the preparation of linear maltodextrins was developed. To utilize the LBCD debranching and ring-opening activities of CMApu, the linear malto-oligosaccharides from maltooctaose to glucose could be prepared from G8-β-CD. To utilize AM-degrading and AP-debranching activities of CMApu, the amylodextrins with a DP range from 1 to 76 could be prepared from normal starch instead of the expensive waxy starch, showing a great potential for industrial applications. And, the high optimal temperature of 95–100 °C for CMApu activity facilitated the simultaneous gelatinization and hydrolysis of cereal starch.

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